A further Contribution to the Study of *Pelomyxa palustris* (Greeff).

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(Plates 36–38, & text-figure.)

The freshwater Rhizopod *Pelomyxa palustris* was first described by Professor Greeff in 1867 as *Pelobius* and later discussed by the same observer under its present name.

Between 1869 and 1879 his researches were followed by those of Leidy and Korotneff. In 1891, Prof. A. G. Bourne studied another species (*P. viridis*) and published his observations on this and the species in question in the 'Quarterly Journal of Microscopical Science.' Prof. Bütschli, in 1892, contributed to our knowledge of the structure of the protoplasm in *Pelomyxa*; and later observers, notably Dr. Pénard in 1893 and since, have published observations of great interest relating to this curious, and in some respects mysterious, Protozoon. The present writer, in a paper published in the 'Quarterly Journal of Microscopical Science' in 1894, brought forward some points with regard to the minute structure of *Pelomyxa palustris*, together with some new observations confirming, as far as they went, the suggestions of Prof. Bourne and Dr Pénard that the rods found scattered through the protoplasm of *Pelomyxa* were neither crystals nor protein crystalloids, as had been previously supposed, but bacteria. Mr. M. D. Hill undertook some culture-experiments with a view to establishing this, but these had negative results.

The view that the rods are bacteria has, however, found general acceptance, though no absolute proof has yet been published. In Dec. 1895 an opportunity offered itself to me for the further study of this and other questions relating to *Pelomyxa*; investigations were then undertaken, and have been continued at intervals each year up to 1903. The results of these investigations are embodied in the present paper, and the new points which it is hoped thereby to establish, with regard (1) to the bacteria, (2) to the refringent bodies, (3) to the general structure and behaviour of the animal itself, are, mainly, as follows:—

The Bacteria.

(1) The definite proof that the rods are bacteria
  (*a*) by their motility and division,
  (*b*) by their reactions,
  (*c*) by successful culture.
(2) The life-history of the bacteria, in fresh preparations and in culture; and
(3) The identification of these as a new species, which it is proposed to name *Cladothrix pelomyxæ*.

The Refringent Bodies.

(1) The proof that the refringent bodies are proteid in nature.
(2) That they have a definite relation to the bacteria, supplying them (*a*) with a point of attachment necessary for the completion of their development, (*b*) probably also with nourishment.

The Animal as a whole.

(1) Some observations on the nature of the pseudopodia, which tend to show that a recent classification of Rhizopoda based on the characters of the pseudopodia, as hitherto known, will not hold good as regards *Pelomyxa*.
(2) Observations on
  (*a*) the division of a single *Pelomyxa*,
  (*b*) the fusion of a divided portion with the protoplasm of a second *Pelomyxa*.

It will be convenient to consider these points in the order given, taking first

THE BACTERIA.

The true nature of these was first definitely established by me when working in my private laboratory in December, 1895. It had appeared desirable to study *Pelomyxa* not only during the summer months, but also during the winter, if possible, and with this view Mr. Bolton was applied to for a supply of living specimens. He was only able to supply seven individuals, reporting them as "scarce at this time of the year." The first question to which I directed my attention was the motility of the rods, as the fact of motion had always been observed in rods

cast out from the living animal; but Prof. Bourne and others had considered this motion to be possibly molecular, and in my previous observations it had not been possible to satisfy myself absolutely that the motion was not due to currents created in the water by the activity of the pseudopodia, or to other physical causes. The seven *Pelomyxæ* used in this investigation were healthy but sluggish specimens, in which rods and refringent bodies were very abundant and the former unusually large and thick. The method of observation adopted was to crush a *Pelomyxa* in a drop of water, so as to set free the bacteria, and to watch these continuously for periods of several hours without intermission (generally for 3 hours in the morning and 2 hours in the afternoon), without removing the eye from the microscope.

Motility.—The rods, when set free, moved at first actively at the free edge of the animal, gradually becoming more sluggish and eventually coming to rest altogether. As a result of continuous observation directed to this one point only, I was able to establish on the first day that the motion was undoubtedly one of translation. The rods moved even very rapidly at times, in opposite directions and in every direction; the *Pelomyxa* having been killed by crushing, there were now no currents due to motion on the part of the animal; further, the drop was not allowed to evaporate, hence there could be no currents due to evaporation.

The motion of the rods was both horizontal and vertical, and could often be seen to change from one plane to the other; several times a rod was observed to swim vertically and then turn over and travel away horizontally. The motion was of the kind always associated with the presence of flagella, and suggested the presence of flagella at each pole, or possibly all round. While watching a large rod of six joints in one of the "pools" made in the interior of the *Pelomyxa* by crushing, it was seen to swim actively in the pool and then to pass *through* an intervening bridge of protoplasm into a second pool. By focussing the rod carefully during transit it was possible to be certain that it passed *through*, not under or over the protoplasm, and this with a peculiar boring action like that of a bradawl when used to make a hole, viz., a revolution through half of a circle and back.

The attention of other observers was called to the motion of the rods, and all testified that it was transitional.

Having thus established satisfactorily that the rods were capable of independent motion of a bacterial nature, attention was next directed to watching for their division.

Division.—It was fortunately possible, by the same process of continuous observation, to establish the fact of division of a rod. For this purpose, however, periods of 3 hours were not enough; the observation required to be absolutely unbroken for the whole day. My thanks are here due to my husband, who rendered continuous observation possible by taking my place at the microscope when short absences were necessary for meals.

The 28th and 29th of December were spent in this manner, and during these two days I observed division several times, a single rod of course being kept under observation in each case. Division took place in the following manner: a rod consisted of two equal joints A B; A formed a third joint by the appearance of a new constriction dividing it exactly at the middle. At this stage the rod presented the appearance of being divided into one long joint and two short ones. Sometimes the newly-made joints rapidly grew to equal length with joint B before further division, and this accounted for the rods with an odd number of joints (three or five) so commonly found. Sometimes, however, joint B followed suit almost immediately with division, resulting in the formation of a rod with four joints of equal length. In this case history repeated itself, viz., either one of the terminal joints divided again (5 joints), or both terminals did so, producing a six-jointed rod. Now, and now only, so far as I was able to observe, did actual separation take place, a 4-jointed rod breaking in half, or a 6-jointed rod breaking off *two* of the terminal joints *in one piece.*

This appears to be absolutely characteristic of the organism under normal conditions, and accounts for the fact that *free* single-cell rods are, as far as my experience goes, never found; hence the necessity of starting from a two-jointed individual, which at first sight seems not to be beginning at the beginning. This division from a double unit surprised me exceedingly, and I am not aware if such a mode of division has any parallel among known life-histories; however, the fact remains that subsequent investigations only served to confirm the observation, and indeed it afterwards formed an important means of identification.

Once an 8-jointed rod was seen to break off four joints, but in no case was the unit set free a single one. Division was by

no means frequent, and had the periods of observation been less protracted it might easily have been missed.

The actual process was very rapid, occupying about one minute; the rod under observation bent and straightened itself alternately about three or four times, with a lashing movement, and then broke at the point of bending (*i. e.* the second joint from the terminal) and each part travelled away, generally in opposite directions, viz., going, so to speak, original pole first, and showing at that end the slight "halo" which seemed to point to the existence of flagella.

Reactions.—The rods, which may now be definitely called bacteria, stained well with all bacterial stains, notably with Gram, with Heidenhain's iron-hæmatoxylin, and with all the aniline stains.

Almost all known stains for flagella, viz., Loeffler's, Van Ermengem's, Fischer's, Moore's, and very many others, or modifications of these, were applied in the hope of demonstrating the presence of organs of locomotion, but though an appearance suggesting either a bunch of flagella, or a single very thick flagellum, at the poles, often resulted, yet the preparations were never sufficiently definite to satisfy me on this point. It is well known that the flagella of certain bacteria (Fischer, 12) may be resistant to all but one particular ingredient in the stain, and I have little doubt that the demonstration of these flagella is merely a question of some such ingredient which has not yet been hit upon, or of greater technical skill in the operator. Other possible reasons for the failure to demonstrate the flagella may be (1) the extreme delicacy of these organs, which often causes them to be thrown off in the preliminary process of fixing, or (2) the fact that they may possibly not be permanent structures at all: appearances at times have suggested both these explanations.

Life-history of the Bacteria.

It still remained to follow out the development of the bacteria, and I was ultimately led to success in this undertaking by a very curious and happy accident which occurred in December 1895, while the seven *Pelomyxæ* already spoken of were under observation, and therefore it will not be out of place to describe this occurrence at this point, although it is also closely connected with work on the refringent bodies which is to be discussed later. On Dec. 30, 1895, on going to procure the last two of the *Pelomyxæ* from the glass vessel in which they had been placed, no specimens

were to be seen, but a very large Rotifer was discovered, which had escaped my notice previously, but now attracted it by an unusually milky-white appearance.

The suspicion that the Rotifer had devoured the missing *Pelomyxæ* was confirmed by microscopic examination, and as no more material could then be obtained, it was determined to tease up the Rotifer in water, in the hope of finding the bacteria and refringent bodies in useable condition. This was done, and the contents of the *Pelomyxæ* were found to be practically unchanged. The refringent bodies separated out in the water, and the bacteria were found to be quite alive, and were seen to be attached to the walls of the refringent bodies in great numbers, and to be in active "wobbling" movement on these. Seen "end-on," they presented such a curious and interesting appearance as to induce continued careful study. In the course of 1½ hour's watching a very curious thing happened; the debris of the Rotifer contained, among other things, a good many Rotifer ova, and the bacteria gradually detached themselves from the walls of the refringent bodies and swarmed upon the ova of the Rotifer instead, and this in such numbers that the ova soon were quite covered and could only be distinguished from the refringent bodies (in the former condition of these) by focussing through to find the nuclei. The refringent bodies were eventually quite deserted, and remained clear and homogeneous. Clearly, then, the ova possessed an attraction for the bacteria, which might be of a purely chemiotactic nature; but on the other hand the bacteria might be attracted to the ova either as a source of oxygen or as a food-supply. It seemed unlikely that bacteria whose natural habitat was in the interior of living protoplasm should be highly aerobic, and therefore the hypothesis that they regarded the ova as a fresh source of food seemed the most likely. At any rate this was taken as a working hypothesis, and the first conclusion to which it led was that the theory which applied to the ova might also apply to the refringent bodies, and that the long-observed relation between these and the bacteria might be that of food-supply. A second inference drawn from the observation was that, if the bacteria were attracted to feed upon albuminous bodies, such as ova, some form of albuminous liquid would be the proper medium in which to attempt their cultivation; and further, that some light was now thrown upon the probable composition of the refringent bodies themselves. Subsequent investigation (in

1896) confirmed these conclusions, as will be explained in the section of this paper dealing more particularly with the refringent bodies.

Meanwhile, with regard to the bacteria, it was by working on these lines that it was eventually found possible to follow the development through all its stages, though it was not until 1899 that success was fully attained.

In the spring of 1896 Mr. Bolton was again applied to for a supply of *Pelomyxæ*, and 28 individuals were obtained. These were chiefly used for work on the refringent bodies, but on March 13 some rough attempts at cultivation of the bacteria were made, using as a medium Mann's twice-filtered dilute solution of egg-albumin in sterilized distilled water. The albumin, being drawn straight from the egg, was tolerably germ-free, and was put into a sterilized tube, to which a *Pelomyxa*, teased up with a sterilized needle, was added, and the tube closed with sterilized cotton-wool, and placed in the same temperature as the vessel containing the supply of living *Pelomyxæ*.

On March 16 the tube was removed to a slightly warmer temperature. In a few days a white filamentous growth was seen to be proceeding from one of the teased-up portions of *Pelomyxa* in the tube, and on microscopic examination was found to consist of a pseudo-branching system, attached to one of the refringent bodies, containing rods in a single-walled sheath, which broke down, while under examination, to form free bacilli.

A preparation was made of this, but, owing to the nature of the medium in which the growth was formed, the preparation showed too much stained deposit around the filaments and rods to be instructive. At the time, also, not much importance was attached to this development, owing to the fact of the albumin not having been sterilized; the growth was regarded as probably belonging to the common species of *Cladothrix* (*C. dichotoma*), which might have been ingested by the *Pelomyxa*, and consequently as vitiating the experiment. It was not realized until later that the actual development sought for had probably been accidentally obtained.

Further experiments at this time gave negative or mixed results, and were beset with the difficulties, formerly experienced by Mr. Hill, of properly sterilizing the animal itself, as well as the impossibility of satisfactorily sterilizing by heat a coagulable medium

The years 1897 and 1898 had to be entirely devoted to another bacteriological research, published in 1898, and it was not, therefore, till the spring of 1899 that attention could again be given to the study of *Pelomyxa*.

In April 1899 the interesting discovery was made at the University Museum, Oxford, of a very large quantity of *Pelomyxa palustris* in a tank in the grounds of the Department of Comparative Anatomy, and my best thanks are due to the Linacre Professor for his great courtesy and kindness in not only placing at my disposal what was practically an unlimited supply of material, but affording me an opportunity of working at this in his laboratory, where most of the following research was accordingly carried out. It may be interesting to note that the colony in the tank must almost certainly have originated from stray individuals accidentally emptied into this tank by me during my researches as a student under Prof. Lankester in 1893. The specimens now found were full-fed healthy individuals, and most were of unusually large size, and contained abundance of rods and refringent bodies; they were generally of a dirty olive-green tint, which, though probably due to food, was found to be characteristic of all really healthy specimens; only starved or sickly ones ever exhibit a milk-white appearance.

It was now determined to undertake a fresh series of culture experiments, side by side with more elaborate study of the bacteria in a great number of fresh preparations, and, if possible, to check the cultures by comparison with stages which might be discovered in the fresh preparations. It appeared that only in this way could one be certain of the correctness of results obtained in culture, as an insuperable difficulty to complete sterilization presented itself during the study of this batch of *Pelomyxæ*. This was the fact, not sufficiently realized either by myself or Mr. Hill on previous occasions when we had only a few individuals to work with, that even if complete sterilization of the outside of a *Pelomyxa* could be effected, a number of bacteria other than the rods in question were always present in the interior of the animal, having been ingested with the food, and these could not possibly be eliminated without killing the rods. Fortunately for the experimenter, these accidental bacilli did not at all closely resemble the rods, being very much finer and thinner; therefore there was no difficulty in identification under the microscope.

The theory I had formed was that single rods might settle

upon refringent bodies as a support, possibly also deriving food from them; if this were the case, it ought to be possible to find some evidence of it, if a sufficiently large number of *Pelomyxæ* could be examined.

After the examination of a great many fresh preparations, in which *Pelomyxæ* were teased up in water and their contents carefully studied, I was fortunate enough to obtain one in which several single filamentous growths occurred, each attached to and springing from one refringent body. They were composed of a single rigid slightly-curved chain of jointed rods exactly similar to the characteristic jointed rod of *Pelomyxæ*; the joints, of which there were 20–30 in a single growth, were of the same thickness and length as the free rods and stained in a precisely similar manner, leaving the fine single-walled sheath colourless and barely visible.

They are figured in Pl. 36. figs. 1, 2, which are reproduced from photomicrographs by Dr. G. Mann. One or two showed pseudo-branching (fig. 1). Later on in the course of my researches, a fully-developed pseudo-branching filamentous growth, attached by its base to a single refringent body, was teased out of a fresh *Pelomyxa* and is figured on Pl. 36. fig. 4. Also on several occasions a live *Pelomyxa* under observation was seen to eject a complete branched system of this kind, the effort to get rid of so large and rigid a body often resulting in the "bursting" or disintegration of the animal.

The branched systems so ejected ultimately broke down to form 2-jointed free rods. A preparation was made of one such ejected growth, which exactly resembled those previously described and figured, and stained well with iron-hæmatoxylin. It seemed clear that I had obtained two of the later stages of development, but the earliest stage, viz., the free unit fixing on a refringent body, had not as yet been found. The observations were extended to a great number of *Pelomyxæ* in the hope of finding this also, and eventually success was attained.

In a teased preparation a single 2-jointed rod (double unit) was seen to fix itself by one end to a refringent body. A minute "blob" of secretion appeared to be formed at the point of attachment, which fixed the rod firmly to its support. (Pl. 36. fig. 5.)

Later, other rods were observed to fix in the same way.

Any slight jolt or vibration occurring before the secretion had become firm was sufficient to dislodge the bacillus, but at a later

stage it appeared to be rigid, and was then much more resistant to shocks of this kind. Having once secured the fact that the bacilli did so fix themselves, I isolated several 2-jointed rods with a few refringent bodies in a drop of water sealed from evaporation by means of soluble glass, and these without exception fixed themselves upon the bodies, but they did not grow, remaining quite stationary as to size and number of joints.

More advanced stages (three and five joints) obtained in other fresh teased preparations are, however, seen on Pl. 36. figs. 6, 7, rendering it evident that under normal conditions growth took place after fixation, for no rod of greater length than the double unit was ever seen to fix itself. Proof of this growth was finally obtained by isolating 2-jointed rods and refringent bodies from a fresh preparation in a hanging-drop of Mann's dilute solution of egg-albumin, when growth occurred, and the rod under observation attained 5 joints, but never developed fully, owing to the highly artificial conditions. Three stages of such growth, as drawn at intervals of 24 hours for three days, are seen in Pl. 36. figs. 8–10. A difficulty was, however, experienced at this stage of the work which unfortunately necessitated my transferring it to another place. The floor of Prof. Weldon's laboratory was found to vibrate so much that the commotion caused in a drop by a passing footstep was sufficient time after time to dislodge bacilli in an early stage of fixing. By the kindness of the Waynflete Prof. of Chemistry I was, however, enabled to set up my apparatus for the hanging-drop cultures above described in a ground-floor room of his department at the University Museum, on a balance-table built up from the foundations, where the vibration difficulty was entirely overcome and success attained.

A number of cultures were now undertaken, but owing to the difficulty of sterilizing egg-albumin, it was suggested to me to use serum-albumin as the medium. Dr. W. Ramsden, of the Physiological Department at the University Museum, kindly provided me with some fresh sheep's serum, which I sterilized by sucking it through a Muncke's filter by means of a vacuum-pump into sterilized flasks which were closed with sterilized cotton-wool. Two *Pelomyxæ*, which had been kept hungry in clean water, in order to minimize as far as possible the danger of contamination from their contents, were then cleansed externally as far as possible by washing them successively in eight vessels of sterilized distilled water. They were then thoroughly

teased up with sterilized needles and "planted," one in each flask, and both were placed in the dark at room-temperature. Both flasks gave abundant growths, of course of a mixed character. From these fresh cultures were made in the same medium, selecting as far as possible those large rods which were identifiable as characteristic of *Pelomyxa*; and this process of selection and re-selection was repeated constantly until an approximately pure culture was obtained (Pl. 36. fig. 3.)

From this nearly pure culture two more flasks were inoculated and gave growths of rods resembling those of *Pelomyxa*, and stainable in the same manner; also a hanging-drop of egg-albumin, containing a few refringent bodies, was inoculated from the pure culture and gave results exactly similar to that inoculated with rods from the fresh preparation above described (Pl. 36. figs. 11–13).

From a comparison of these figures it will be admissible to consider the growths as identical. In the drop the rods had fixed on the refringent bodies; in the flasks they fixed both on refringent bodies and on the walls of the flask, and, growing very slowly, attained a considerable length, but in neither culture did they show at this time any sign of branching. After a growth of a fortnight, they exactly resembled those unbranched filaments first discovered in *Pelomyxa* growing on the refringent bodies.

At this time I was obliged to be away from home for six weeks, leaving a pair of flasks with cultures two weeks old in the condition described. When I returned, I found that the filaments in these cultures had branched, and that the branching was of the pseudo-dichotomous kind previously observed in the fresh preparations, viz., either the kind of apposition commonly known as peculiar to the genus *Cladothrix*, or that early stage of it, a dichotomous branching of the *sheath only*, which has been figured by Fischer for *Cladothrix* (Pl. 37. figs. 14–16.)

From the nature of the medium in which the filaments were grown, it was almost impossible to make a satisfactory permanent preparation of them. But it seemed so desirable to preserve some evidence of the branching that my friend Dr. Gustav Mann, who was consulted with regard to this, kindly attempted to make a preparation for me of the bacteria from the serum by a special method of precipitating them.

His ingenuity produced a fairly successful preparation, sufficient to show the point in question, although the method

used created a crinkled and unnatural condition of the filaments. The filaments in the other flasks, which contained older cultures, had, on my return, already broken down to form short rods, so that the cycle had been completed without my being able to state at exactly what period this breaking-down had taken place. It will be seen, however, that the stages observed in (1) the fresh condition, (2) in cultures, were five in each, and that, as the following table shows, the stages in (1) were exactly parallel and similar to those in (2) and may fairly be considered identical.

	Fresh Preparations.	*Cultures.*
Stage	1. Motile 2-jointed rods (units).	1. Motile 2-jointed rods.
„	2. Fixing of unit on refringent bodies.	2. Fixing on solid bodies.
„	3. Growth to form long filaments.	3. Growth to form filaments.
„	4. Pseudo-branching of filaments.	4. Pseudo-branching of filaments.
„	5. Breaking-down to form short rods.	5. Breaking-down into short rods.

Although the circumstances of this case necessitated a mode of procedure necessarily less exact than that justly demanded in most bacteriological experiments, yet the above stages which have been observed and recorded form a complete cycle of development; and the conclusion reached must surely be, that the life-history of the bacteria characteristic of *Pelomyxa* has been placed beyond reasonable doubt.

Identification of the Bacteria.

The classification followed is that of Migula. The order is that of Eubacteria. From the character of the sheathed filament, and the reproduction by "swarmers" set free and immediately recommencing the cycle, the bacteria of *Pelomyxa* belong to the family Chlamydobacteriaceæ.

From the single contour of the walls of the sheath, from the equal size of the filament from base to tip, and from the pseudo-dichotomous branching, the bacteria belong to the genus *Cladothrix*, Cohn=*Sphærotilus* (Kützing, Mig.). Only two species of this genus are known—viz., *Sphærotilus dichotomus* (*C. dichotoma*, Cohn), and *Sphærotilus natans*, Kützing. The species in question differs from both in one important particular, viz.—the free rods or swarmers of *Pelomyxa* are straight and always most distinctly and sharply truncated at the ends,

whereas the swarmers of *C. dichotoma* often slightly tend to be kidney-shaped and are always rounded at the poles; the swarmers of *S. natans* are also slightly rounded at the ends. Both these species occur as tufts composed of several filaments whether fixed or free; the bacteria of *Pelomyxa* appear to occur exclusively as single filaments. In size the rods agree fairly with either species, the average thickness being 2μ. They resemble *S. natans* very closely in the slight separation of the cells in the filament from each other (shown very distinctly in stained preparations); but differ in the fact of the *free* unit always being double and in the greater rigidity of the filament as a whole. The habitat must be taken into consideration, with the fact that, as far as my search went, neither of the known species of *Cladothrix* occurred in the tank whence the greater number of *Pelomyxæ* were taken; and, further, that as far as our knowledge extends at present, there is no evidence that the bacteria of *Pelomyxa* can live for any length of time apart from their host. On the whole, then, it seems justifiable to regard them as a new and distinct species of *Cladothrix*, which it is proposed to name *Cladothrix Pelomyxæ*, and to classify as follows:—

Order, Eubacteria.
Family, Chlamydobacteriaceæ.
Genus, *Cladothrix*, Cohn; *Sphærotilus*, Kützing.
Species, *C. Pelomyxæ*.

The Refringent Bodies.

Some reactions of the refringent bodies had been tested by Greeff and others, but further experiments were made in the course of the present investigation. They were tested for starch with a very dilute solution of iodine, but gave no reaction whatever. (With *strong* iodine a brown colour was obtained, as previously recorded by Greeff.) Starch was therefore excluded. Paramylum is unstained by dilute iodine, hence the refringent bodies were next tested for this; their behaviour with strong sulphuric acid also suggested a similarity, but their resistance to dilute potash excluded paramylum. They were also compared with the concretions in *Lithamœba*, as studied by Prof. Lankester; the reactions were found very much to resemble those recorded for *Lithamœba*.

The occurrence above related in connection with the bacteria and the ova of Rotifer now gave a new direction to experiments.

With a view to ascertaining whether the bodies were albuminous, or albuminoid, almost every known micro-chemical test for albumins was applied; these tests are not numerous, and most are not decisive, but were found to give the reaction, said to be characteristic, equally well with the refringent bodies and with almost anything else, *e. g.* the fingers of the operator, and even with blotting-paper. The most reliable, however, appeared to be (1) that with Millon's Reagent, (2) the violet reaction with sugar and sulphuric acid, (3) the xanthoproteic test, and (4) that with caustic soda and copper sulphate. As far as my control experiments went, none of these gave the reaction with wrong substances.

The Nature of the Refringent Bodies.

The effect of these reagents as applied to the refringent bodies was as follows, used on freshly-teased up *Pelomyxæ* taken from water. On application of Millon's reagent there was no staining at first, either of the protoplasm or of the refringent bodies. Then a milky precipitate occurred, with coagulation of the protoplasm, followed by the appearance of a yellowish tinge, and the gradual disintegration both of the protoplasm and the refringent bodies; the latter collapsed, became nodular (Pl. 37. fig. 17), and ultimately dissolved. On warming, an hour after the addition of the reagent, a delicate pink colour was observed in the refringent bodies, and also in the protoplasm, vanishing on cooling. If heat was applied *immediately*, a deep pink colour was obtained, but the bodies dissolved almost at once. Heating immediately, but less strongly, gave a good pink colour, and the refringent bodies only gradually disintegrated into nodules and disappeared. The depth of colour evidently depended upon the degree of heat applied immediately, but, if heated over a certain point, the destruction of the bodies was too rapid to allow of the reaction being observed.

With strong sulphuric acid the refringent bodies were dissolved; sugar solution, run in afterwards, gave a very distinct, but pale, violet coloration of the liquid.

Strong nitric acid coagulated the protoplasm and shrivelled everything up, but gave a pale yellow colour on heating, deepening slightly on addition of ammonia. A 1% solution of caustic soda, followed by a very dilute solution of copper sulphate, gave no violet coloration even on heating.

The reactions with three out of four tests were, then, perfectly definite, and the conclusion reached was that the refringent bodies are certainly proteid in nature, and are probably some form of albumin, the product of metabolism in the protoplasm of the animal.

It may be mentioned that the reaction with picric acid in turpentine described in my previous paper in the 'Quarterly Journal of Microscopical Science' is consistent with those obtained as above, since the peculiar "bright crescentic areas" produced in the refringent bodies by that stain only, may have been the "local bead-like coagulations" said to be given when crystals of picric acid are dissolved in solutions containing albumin.

The Relation of the Refringent Bodies (a) *to the Bacteria*, (b) *to* Pelomyxa.

(*a*) That the refringent bodies certainly afford to the bacteria a point of attachment without which the cycle could not be completed has been already explained. It seems, however, most probable that the relation is a twofold one, and that the refringent bodies serve them also as a food-supply. For the bacteria are found to settle upon the refringent bodies in numbers, in preference to other solid bodies of which plenty are always accidentally present in the protoplasm of *Pelomyxa*; growth has been proved to take place while this relation continues: further, the facts that the bacteria swarmed upon the albuminous ova of Rotifer in exactly the same manner as on the bodies, and that they have been proved to live and flourish in albuminous media, lend great support to this view.

(*b*) The relation of the refringent bodies to the *Pelomyxa* as a whole is extremely difficult to determine, and must perhaps remain an open question. Two views are possible, and either is rather plausible:—(1) the refringent bodies may be a reserve food-supply to the animal itself; in this case one would expect it to be drawn upon when other food was unobtainable. Several experiments were undertaken with the object of testing this: many *Pelomyxæ* were kept in clean water without mud or food of any kind, but none survived more than a few days, and in these the refringent bodies were *not* diminished in size; in control experiments in which *Pelomyxæ* were kept in clean

solutions of egg-albumin without mud, they lived well and divided—a result which might cut both ways.

If, however, the bodies act as a reserve food-supply to *Pelomyxa*, it must be a distinct disadvantage to the animal to have an almost unlimited number of bacteria drawing upon the same supply, and thus we should be left without any explanation of the relation of the bacteria to the animal. It would also be inexplicable that *Pelomyxa* should constantly eject refringent bodies, as it normally does.

(2) A second view, viz., that the refringent bodies are a waste product of metabolism, useless to the animal, seems, therefore, more tenable. In such a case the presence of the bacteria would be perfectly explicable, as they would be of direct advantage to the animal in clearing off useless products, while the proved ejectment by *Pelomyxa* of the bacteria, when the branching system became so large and rigid as to be inconvenient, would also be an advantage to the bacteria, in enabling them to scatter their swarmers so as to be ingested by fresh hosts with the mud of their habitat.

The condition of the resting or quiescent *Pelomyxæ*, which had filled themselves up with sand, described in my previous paper, is very consistent with this view; for in some of these the refringent bodies were very few and very small, and in others practically non-existent, and the bacteria were equally scarce. This suggests that metabolism not being then active, no waste products were being formed and no scavengers were required; of course it might also mean that the reserve supply had been already exhausted, but if that were the case it is difficult to imagine what the animal could subsequently have been living upon.

Taking all the circumstances into consideration, the view of the writer is that the refringent bodies are waste products of the metabolism of *Pelomyxa*, and that they serve in a double sense for the support of the symbiotic bacteria.

The Animal as a whole.

Several points of interest as to the general behaviour of *Pelomyxa palustris* were brought out in the course of these investigations: the first of these refers to the character of the pseudopodia, which are generally described as lobose and blunt.

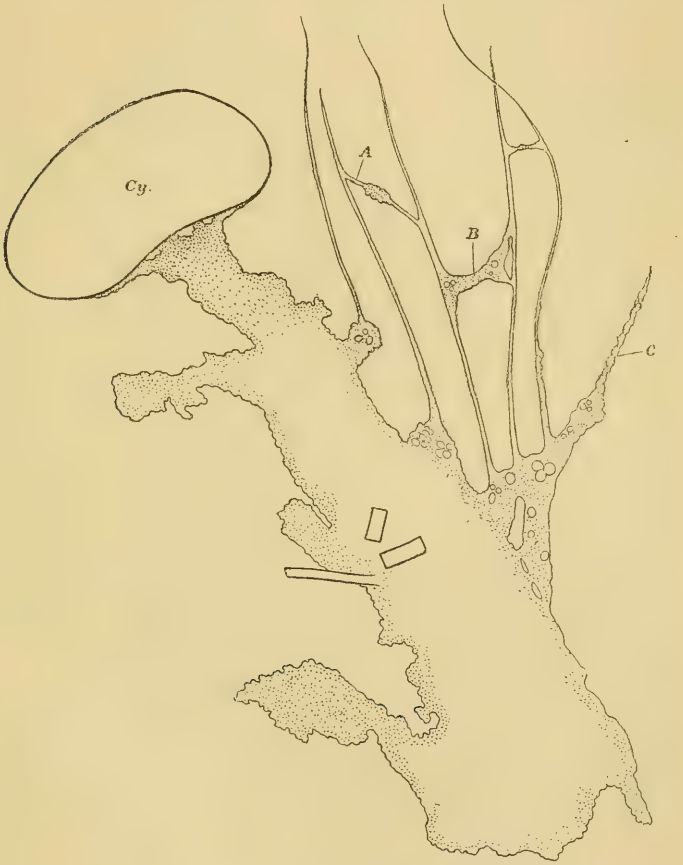
This appeared to me, from a study of many individuals, to be only the case when the animal is sluggish or creeping very quietly; at such a time the contour is certainly perfectly even, and the pseudopodia are as described, showing the hyaline border very clearly (Pl. 37. fig. 18). Under circumstances equally normal, but inducing greater activity, as when a portion is constricted off naturally, or the animal is getting rid of a large solid or rigid body, the contour frequently becomes temporarily quite ragged, and whip-like pseudopodia of exceeding fineness are shot out with great suddenness and velocity, extending to a considerable length (p. 391). Pseudopodia of this kind are exceedingly attenuated and acute, and are, for a great part of their length from the tip inwards, perfectly hyaline, appearing to be actual prolongations of the hyaline border; they often, but by no means always, radiate outwards, and very frequently anastomose, the connecting bridge between two pseudopodia being sometimes hyaline (p. 391, *A*), but more often consisting of fine strands of granular protoplasm (p. 391, *B*). They are never rigid, and often fall into the most graceful curves. When one of these fine pseudopodia is in course of being retracted, a very curious feature is observable, viz., a peculiar wrinkling of the granular part of the protoplasm, as if it were a soft coating to an elastic core (p. 391, *C*), yet no such actual distinction can be microscopically detected, although the withdrawal of a pseudopodium is a far slower process than its extension.

This observation would appear somewhat to vitiate the classification of Rhizopoda recently proposed by Professor G. S. West, which is founded chiefly on the nature of the pseudopodia, and in which it is stated that those of the order Amœbœa, to which *Pelomyxa* is assigned, are "rarely attenuated and acute, sometimes branched, but never reticulate." It appears to the writer that pseudopodia in general are too transient characters on which to base a classification, and that those of *Pelomyxa* in particular do not agree with the description proposed by Prof. West in his interesting paper.

Prof. West's suggestion that the "characters of the nucleus" in Rhizopods are "of much less importance in these animals than might at first be imagined" was borne out as far as my experience went. In the many experiments made by me with living *Pelomyxæ* in nutrient fluids, when they very commonly divided, the nuclei were always examined for any characteristic

appearance at the time of division, but they were always in a "resting" state, and presented no appearance suggesting that they initiated or took an active part in division.

Division, whether affecting only a small portion of the animal, or one-third or one half, appeared to be always of the same simple



Pelomyxa palustris extruding *Cypris*-shell, with production of acute whip-like pseudopodia. *A*, hyaline connecting strand; *B*, granular do.; *C*, pseudopodium in the act of retraction; *Cy.*, *Cypris*-shell.

character, and generally to be induced by unfavourable conditions, such as drying-up of the liquid surrounding the animal; the fragments form perfect individuals in quite a short time, and obviously several small individuals would have a better

chance of survival under unfavourable conditions, or in a diminished supply of water, than a single large one. Large individuals have a tendency to burst, and I am in agreement with Pénard's latest view (1902) that this is a quite frequent and normal occurrence, and not due to surface tension or damage. Once, in the whole course of the investigations, a single torn and damaged *Pelomyxa*, "planted" in a flask of egg-albumin stoppered with sterilized cotton-wool, gave rise to a very large number of amœbæ, each possessing a single nucleus and contractile vesicle; but as this production unfortunately did not take place under actual observation, the fact only can be recorded. Such observations have been made before, by Greeff and Korotneff, but in view of their scarcity, and the fact that they have never received general acceptance, it seems worth while to note this, and also to mention that the circumstances excluded all possibility of any other origin for the amœbæ, with which the liquid literally swarmed. Pénard has noted a similar occurrence, since Greeff and Korotneff. One other curious point must be recorded. Section had been made of two *Pelomyxæ* into two pieces each, and these four fragments A^1, A^2, of one individual, B^1, B^2, of another, were watched under the microscope in one field, those belonging to one individual being kept on the left, the others on the right. (This was being done with a view to observing whether the ragged pieces rounded up again as separate individuals, as noted by Pénard for *Difflugia*.) A^1 constricted off a further piece A^3; A^3, after complete separation from A^1, made independent movements, and in so doing approached B^1.

A^3 and B^1 came into contact, and A^3 immediately fused with B^1, becoming in a few moments quite indistinguishable from it (Pl. 38. figs. 22–24). A similar behaviour of fragments has been recorded by Pénard for *Difflugia*, but he expressly stated that, while a fragment readily fused with another *recently severed* fragment *from the same individual*, no such thing ever occurred between fragments from different individuals.

This observation with regard to *Pelomyxa* appears, therefore, to be new, and may possibly have considerable significance, since it raises the old question whether *Pelomyxa* can ever justly be regarded as a single individual. Of course it may simply mean that the protoplasm of *Pelomyxa* is chemically homogeneous to an extraordinary degree; but, on the other hand, if fusion of

individuals, or parts of individuals, takes place at all in nature, it would appear that *Pelomyxa* should rather be regarded as a plasmodium. The single nucleus observed in the amœbæ and the multinucleate condition of *Pelomyxa* are facts consistent with such a theory.

Summary and Conclusions.

The conclusions arrived at in this investigation may, then, be briefly summarized as follows :—

(1) The rods are symbiotic bacteria, which complete their development within the protoplasm of *Pelomyxa* and are then ejected, breaking down into free "swarmers," which are ingested by other *Pelomyxæ*, and recommence the cycle.

(2) The refringent bodies are proteid in nature; they consist of some form of albumin, which is probably a waste product of the metabolism of *Pelomyxa*; they have a twofold relation to the bacteria, supplying them with a point of attachment necessary for their development, and (probably) also with a source of nourishment.

(3) The pseudopodia of *Pelomyxa* are not always blunt and lobose, but often exceedingly attenuated and acute, are often reticulate, or anastomosing, and of a different character from any hitherto described for this animal. Classifications based on the lobose nature of the pseudopodia are hereby invalidated.

(4) The division of *Pelomyxa* is of a simple character, in which the nuclei do not play an important part. The only other form of reproduction observed has been, in a single instance, the production of amœbæ, whereby the observations of Greeff and Korotneff are partly, and those of Pénard entirely confirmed.

(5) Under certain circumstances a portion of one *Pelomyxa* may fuse with the protoplasm of a portion of a second *Pelomyxa*, the inference from this observation being that it may prove necessary to regard *Pelomyxa* as a plasmodium.

Bibliography.

(1) Greeff, R.—Archiv für mikroskopische Anatomie, Bd. x. 1879, p. 51.

(2) Leidy, J.—Fresh-water Rhizopods of N. America, 1879.

(3) Korotneff, A. A.—Archives de Zoologie Exp., vol. viii. 1879-80, p. 467.

(4) Bourne, A. G.—Quart. Journ. Microscopical Science, vol. xxxii. 1891, p. 357.
(5) Bütschli, O.—Mikroskopische Schäume, 1892.
(6) Pénard, E.—Archives des Sciences physiques et naturelles, t. xxvi. 1891, p. 134. *Ibid.* t. xxix. 1893, p. 165.
Revue Suisse de Zool., t. vii., 1899; t. viii., Jan. 3, 1900; t. ix., Jan. 2, 1901.
Mém. Soc. phys. et d'hist. nat. Genève, 1890.
Faune Rhizopodique du Bassin du Léman. Geneva, 1902.
(7) Kützing, F. T.—Linnæa, viii. 1833, p. 385; Species Algarum (1849), p. 147.
(8) Eidam, H.—"Ueber die Entwickelung des *Sphærotilus natans*." Jahresber. Schlesisch. Ges. f. vaterl. Kultur, 1876, p. 133.
(9) Cohn, F. J.—"Untersuchungen über Bakterien, II." Beitr. z. Biologie, Bd. i. 1875, Heft 3, p. 185.
(10) Büsgen, M.—"Kulturversuche mit *Cladothrix dichotoma*." Ber. d. Deutschen bot. Ges. 1894, p. 147.
(11) Zopf, F. W.—Zur Morphologie d. Spaltpflanzen, 1882.
(12) Fischer, Alfred.—Untersuchungen über Bakterien, 1897, pp. 3, 23, 94.
(13) Pringsheim, N.—Jahrb. f. wissensch. Botanik, Bd. xxvii. 1895, Heft i.
(14) Migula, W.—System der Bacterien. Jena, 1900.
(15) Rhumbler, L.—Zeitsch. f. w. Zool. vol. xi., 1896.
Biolog. Centralblatt, Bd. xviii.
Archiv f. Entwickelungsmek., Bd. vii.
(16) Prowazek, S.—"Amöbenstudien." Biolog. Centralbl., Bd. xvii., 1897.
(17) Lankester, E. R.—Quart. Journ. Micr. Sci. vol. xix. 1879, p. 484.
(18) Gould, L. J.—Quart. Journ. Micr. Sci. vol. xxxvi., n. s. 1894, p. 298.
(19) Schultze, Franz.—"Rhizopodenstudien, IV." Arch. f. mikr. Anat., Bd. xi.
(20) West, G. S.—"Observations on Freshwater Rhizopods." Journ. Linn. Soc., Zool. vol. xxix. p. 108.
(21) Verworn, Max.—Psycho-physiologische Protisten-Studien. Jena, 1899.
(22) Topsent, E.—Arch. de Zool. Exp. 2^{me} série, t. x. p. xxxi (1892).

EXPLANATION OF THE PLATES.

(Magnification 1000 diameters, except where otherwise specified.)

Plate 36.

Fig. 1. Filaments as outgrowths from refringent bodies. Stained safranin; magnification about 300.

2. Proximal portions of the same two filaments. × 1000.

3. Rods in culture used as starting-point. About × 500.

4. Fully-developed pseudo-branching system attached to refringent body (fresh preparation).

5. Two-jointed rod fixed on refringent body (fresh prep.).

Figs. 6–7. Three- and five-jointed rods on refringent bodies (fresh prep.).

8–10. Three successive stages in development of single rod, in drop-culture inoculated from fresh prep.

11–13. Three successive stages in development of single rod, in drop-culture inoculated from culture.

Plate 37.

Figs. 14–16. Pseudo-branching filaments from culture 8 weeks old.

Fig. 17. Nodular appearance presented by refringent bodies after treatment with Millon's reagent.

Figs. 18–21. *Pelomyxa palustris* dividing off three successive portions, in egg-albumin; normal simple division.

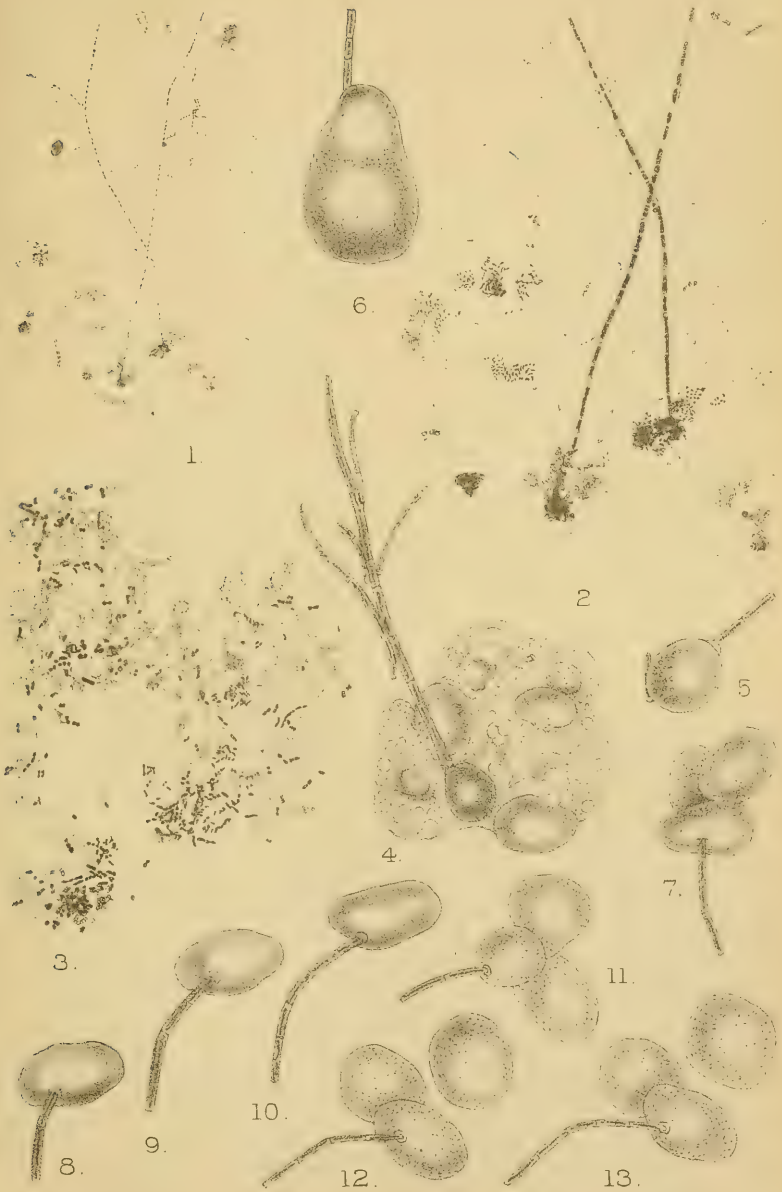
Plate 38.

Figs. 22–24. Division, and subsequent fusion with another individual.

(1) A^1 dividing-off a portion A^3.

(2) A^3 independent, approaching B^1, a portion of second *Pelomyxa*.

(3) Fusion of A^3 with B^1, and withdrawal of A^1.



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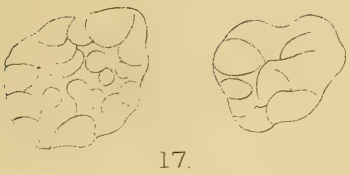

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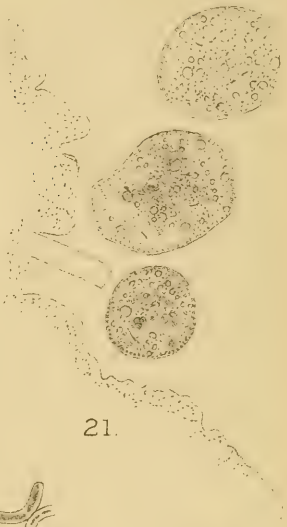

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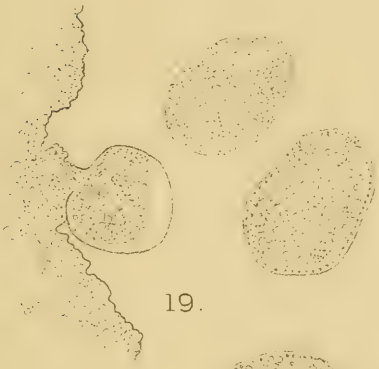

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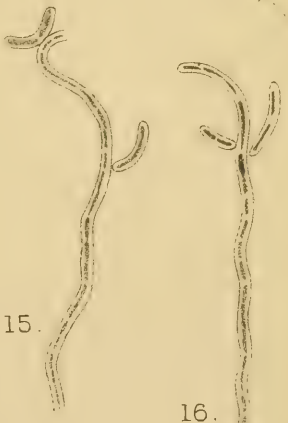

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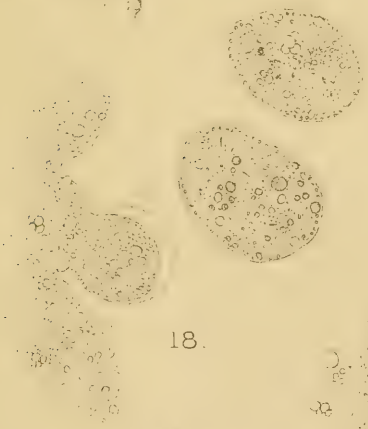

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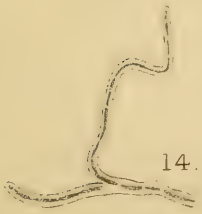

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